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THE RESTORATION OF
THE ACTION POTENTIAL BY THIAMINE

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Joseph Eichenbaum
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7 May 73
Date

THE RESTORATION OF THE ACTION POTENTIAL BY THIAMINE

Submitted by Joseph Walter Eichenbaum, B.A., A.A.

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Last, but not least, I wish to thank my dear wife, Annette, for her technical assistance many nights in the laboratory as well as her abiding moral support.

ABBREVIATIONS USED IN THIS TEXT:

TPP	Thiamine Pyrophosphate or Thiamine Diphosphate
TPP	Thiamine Triphosphate
TMP	Thiamine Monophosphate
ATP	Adenosine Triphosphate
ADP	Adenosine Diphosphate
SNE	Subacute Necrotizing Encephalomyelopathy

ABSTRACT:

Desheathed rabbit vagi were irradiated with an ultraviolet lamp while being superfused with oxygenated Locke solution, until the action potential disappeared. Fluorometric assays for thiamine content in irradiated nerves in parallel experiments indicated negligible levels as compared to those determined for the non-irradiated, desheathed rabbit vagi. One vagus nerve of the pair was then placed in a solution of thiamine and Locke after which it consistently regained its action potential within about 1.5 hours. The second nerve, remaining in regular Locke solution, never regained its conductive activity.

INTRODUCTION:

In approaching the problem of conduction in nervous tissue, two main aspects have grown to influence the thinking of investigators. The first involves the nature of ionic movements. The second, as yet more obscure, consists of the energy considerations which are involved in restoration of ionic balance after the passage of impulses along an axon. Through electrophysiological and pharmacological experimentation, the inter-relationships of the two processes and the behavior of the membrane and its constituents have suggested various mechanisms, e.g., a carrier molecule which is involved in the Na-K ionic shifts between membrane and extracellular fluid and a Na-K ATPase pump which maintains a high K but low intracellular Na concentration in the neurone's resting state.

However, in addition to such postulated mechanisms, a number of findings of concomitant phenomena seem to challenge our understanding of the actual details in the course of events in nerve conduction. One significant group of recent findings implicate vitamin B₁ in this sequence of events. This role of thiamine has been suggested to be a neurophysiological one quite distinct from its well-known function as a coenzyme; i.e. drastic alterations in nerve conduction do not apparently always reduce thiamine-dependent metabolic enzyme levels (transketolase and pyruvate dehydrogenase and alpha-ketoglutarate dehydrogenase) below physiological ranges. For example, an anti-metabolite of thiamine, pyrithiamine, which produces polyneuritis in vivo, alters the electrical activity of isolated nervous tissue by displacing thiamine from the nerve membrane. However, the activity of the thiamine-dependent enzymes remained unchanged. Thus, the question

arises: how critical then is the presence of thiamine to the entire process of nerve conduction? That is to say, apart from its already defined metabolic role, of what significance is its neurophysiological role? This, essentially, was the problem we addressed ourselves to.

HISTORICAL BACKGROUND:

When it was discovered that nerves were sensitive to ultraviolet (UV) irradiation, it was theorized that absorbed photons produced chemical lesions which disrupted the normal sequence of "free energy transfer".^{2,3} Using UV light then as a tool, one could attempt to implicate some of the putative neurochemicals underlying the process of nervous tissue conduction.

Audiat² in 1931, first investigating the effect of UV light on peripheral nerves, found that when he irradiated a whole nerve while in a bath of Ringer's solution, there was an increase in the minimum threshold voltage required for excitation. Simultaneously the action potential amplitude decreased and gradually disappeared. This effect was reversible and the time needed to restore excitability depended on the time and the intensity of irradiation. When he used a filter which absorbed all wavelengths below 310 m μ , no effect on the action potential was obtained.²

The work of Hutton-Rudolph³ with a single motor fiber generally corroborated the discoveries of Audiat in whole nerves. UV irradiation increased the minimum excitability at the node quite rapidly. But excitability ceased within two to eight minutes. When the internodal region of the fiber was irradiated, there was an initial phase of

"over excitability" with a decrease in the required minimum threshold voltage, which then again rose rapidly. In this case, excitability was curtailed after fifteen to twenty minutes. Again, with the use of filters to pass wavelengths greater than 300 m μ these effects were not observed. Maximum effect was obtained with wavelengths below 280 m μ .³

In 1950 Booth et al.¹ studied the relationship between wavelength and intensity of monochromatic UV light producing these effects. They found that only UV light below 320 m μ had photochemical action on nodes in the frog sciatic preparations. Each wavelength studied below this value had its specific activity and the activity curve between 320 m μ and 248 m μ had three apparent maxima: at 297 m μ , 285 m μ and 265 m μ . They pointed out that their results could be discussed in light of the theory of saltatory transmission and K/Na exchanges. They stated that it was probable that the substances in the node which were photochemically affected were related to a Na⁺ shift during excitation. Thiamine might be one of them.¹

It has been demonstrated that thiamine has an absorption maxima at 265 m μ and is destroyed by UV light at this wavelength in about one half hour. This finding has been employed for the destruction of thiamine in various tissue preparations.³⁶

Bachoffer,²⁵ who studied the electrophysiological effects of UV irradiation on single, isolated nerve fibers of the earthworm found that the initial enhancement of activity (increases in conduction velocity, spike amplitude, and rate of rise of spike) was not due to the synergistic action of UV energy and that of the nerve fiber;

nerves continued to respond in an enhanced manner without concomitant irradiation. He concluded that "UV light produces a change in the nerve which is not reversible, at least not without further treatment".²⁵

Von Muralt³⁷ showed a significant difference in UV absorption at 220 and 265 m μ between extracts from excited and unexcited nerves. Extracts from excited nerves contained a greater amount of absorbing material and therefore manifested higher peaks at these wavelengths.

A relationship between thiamine and the nervous system goes back to Eijkman³⁸ and Grijns³⁹. They recognized that it was the absence of a certain dietary factor in rice bran which produced avian polyneuritis and beri-beri. A number of experiments of more recent years have pointed out a possible distinct neurophysiological role for thiamine quite apart from its coenzyme function. Minz⁴ in 1938, von Muralt³⁵ in 1947, Gurtner⁵ in 1961, and Cooper et al.⁶ in 1963 have all demonstrated in a variety of nervous tissue preparations that electrical stimulation results in the release of the vitamin. An antimetabolite of thiamine, pyrithiamine, which produces polyneuritis in vivo, has been shown by Kunz⁷ and Armett and Cooper⁸ in vitro to alter electrical activity of isolated nervous tissue as mentioned earlier. In this latter case, the action of the antimetabolite was to displace thiamine from the nerve, rather than serve as an inhibitor of the thiamine-dependent metabolic enzymes.^{9,10}

The polyneuritis resulting from administration of a thiamine deficient diet is mimicked by the administration of pyrithiamine.³⁰ One of pyrithiamine's analogs, which is ten times more potent in producing polyneuritis in animals, has also been shown to be at least ten times more potent than pyrithiamine in producing bizarre electro-



physiological effects on the vagus nerve.⁸ Oxythiamine and other antimetabolites of thiamine which do not produce polyneuritis in vivo, however, did not have any effect on the action potential or post tetanic hyperpolarization in whole bundle nerve fibers of rabbit vagi.⁸ Apparently, the polyneuritis associated with beri-beri is related to the effect of the pyrithiamine antimetabolite group. Similarly, the polyneuritis related to a dietary deficiency of thiamine does not always correlate with inhibition of pyruvate dehydrogenase, alpha-ketoglutarate dehydrogenase, or transketolase.¹¹⁻¹⁴ This would also suggest an additional, non-metabolic role for thiamine in nervous tissue conduction.

Based on the above studies Tanaka and Cooper¹⁵ modified a procedure for the fluorescence histochemical localization of thiamine developed by von Murralt.²⁶ In various peripheral nervous tissue preparations they reacted freeze-dried preparations with cyanogen bromide and ammonia to convert thiamine to thiochrome, the fluorescent product. With this fluorescent microscopic technique, thiamine was shown to be localized only in nerve membranes and not in axoplasm.¹⁵ In the squid axon, Nachmansohn and Steinbach³² using an enzymatic assay for TPP found TPP to be primarily within the sheath rather than in axoplasm.

Subsequent investigation has revealed an enzyme, TPPase, with an absolute specificity for TPP as substrate among thiamine phosphates and which catalyzes the hydrolysis of TPP to TMP. The enzyme has been specifically localized by electron microscopy in membrane structures.^{16,17} Studies of Novikoff and Goldfischer³³ have shown that TPPase is localized in Golgi apparatus and is currently a marker for this structure.

While the major portion of thiamine in nervous tissue is in the form of TPP, some 4-10% is comprised of TTP. This form of the vitamin has received considerable attention recently because of its complete absence in a post mortem examination of patients with the fatal genetic neurological disease, SNE, in contrast to the normal brain. An enzyme system catalyzing the synthesis of TTP and ADP from TPP and ATP has been isolated from rat brain.¹⁹ This phosphoryl transferase is specifically inhibited by blood, spinal fluid, and urine extracts from patients with SNE.¹⁸

In the face of the accumulated information, and their most recent work, Itokawa and Cooper¹⁹ theorized that presumably this same TTP might serve in the process of conduction in nervous tissue (rather than as a neurotransmitter) by virtue of its relationship to an alteration in membrane permeability during excitation. Rats and frogs were injected with ³⁵S-thiamine and subsequently spinal cords and sciatic nerves were isolated and perfused. They monitored labeled thiamine efflux from the perfused nerve preparations subsequent to the addition of neuroactive drugs in an attempt to ascertain this activity. Acetylcholine, tetrodotoxin, ouabain, and lysergic acid diethylamide all released thiamine. Agents such as choline and sodium chloride had no effect. In brain subfractions this neuroactive drug specificity was particularly striking. The vast majority of labeled thiamine was found in the mitochondrial fraction of the brain with the membrane fraction comprising only about 10%. Yet acetylcholine and tetrodotoxin released thiamine essentially from the membrane fraction and had virtually no effect on mitochondrial-bound vitamin. Trypsin and snake venom, which served as non-specific agents, however,



were found to release about 15% of the labeled thiamine from each subfraction, regardless of whether it was in membrane, synaptosomes, or mitochondria.³⁴ In this same work, the released material consisted mainly of free thiamine and TMP. Also, after electrical stimulation of a peripheral nerve, only the release of free thiamine and TMP has been observed.⁵ Similarly, in some earlier work von Muralt had postulated a "shift from bound to free thiamine" subsequent to electrical excitation.³⁷ Thus these drugs that cause a change in ion movements in nerve, as well as electrical stimulation, also are associated with the dephosphorylation of thiamine phosphate esters.

The fact that sodium chloride and choline were ineffectual in releasing thiamine confers a certain specificity to neuroactive drugs which were able to cause a change in ion movements. Kunz,⁷ who used pyritthiamine to partially inactivate the sodium transport system, also pointed out the apparent connection between sodium movement and thiamine in nervous tissue. In contrast, Petropulos,²⁴ who employed a "complex forming" type of thiamine antimetabolite, whose effect was reversible by addition of excess thiamine in single myelinated nerve fibers from frog sciatic, postulated that the action of the antimetabolite was to decrease the number of active Na carriers. He showed that the height of the action potential was reduced after the addition of antimetabolite. He theorized that since the effect of the antimetabolite was reversible, a loose carrier mechanism involving thiamine and sodium was possible.²⁴

In view of the finding that the dephosphorylation process accompanies the release of thiamine from the membrane, Itokawa and Cooper³⁴ suggested that either (1) ion movement is directly coupled



to the dephosphorylation of TTP or TPP; or (2) that ion movements somehow displace TPP or TTP from the membrane where it undergoes hydrolysis. As the evidence stands to date it seems likely that thiamine plays a role in nerve membrane transport, albeit unclear as yet.

In line with these findings of the destructive power of UV light on thiamine in nervous tissue preparations and the effect of UV light on the action potential, we thought it would be of interest to produce a photochemical lesion in a nerve with UV light, depleting it of its thiamine, and destroying its action potential. Then, we might observe if subsequent replacement of thiamine could restore the action potential.



MATERIALS AND METHODS:

Rabbits were killed by an overdose of ether anesthesia and the vagi were rapidly dissected. A length of about 50-60 mm was excised.

Immediately after removal, the vagi were suspended in oxygenated Locke solution having the following composition in mMoles/liter: NaCl, 156; KCl, 5.6; CaCl₂, 2.2; D-glucose, 5.0; Tris or phosphate buffer at pH 7.0, 2.0-8.0.

The nerves were desheathed according to a procedure of Armett and Ritchie.²¹ Under a dissecting microscope, magnification 30x, the nerve was stretched out in the bottom of a large plastic petri dish containing Locke solution. The nerve was fastened to the dish by small bulldog clamps overlying the thread coming from the tie at each end of the nerve. The entire nerve near the tie was circumscribed with fine tweezer points and delicately the sheath and nerve fiber bundles were separated. A fine, sharpened eye scissor then gently cut along the margin between the retracted sheath and nerve bundle along the length of the nerve in its entirety.

Action potentials were then recorded diphasically from whole bundles of nerve fibers in a chamber similar to that used by R. M. Eccles.²² The nerve was placed in a chamber over five platinum electrodes (two for stimulating, one for ground, and two for recording). Locke solution was infused over the nerve and the electrodes before a slide cover, rimmed about its periphery with an air tight gel, was placed over the chamber.

According to the findings of Evans and Murray,²³ only 13% of the fibers of the cervical vagi are myelinated. Thus, we were primarily



working with nonmyelinated C fibers. The electrical stimulus used was supramaximal for the nonmyelinated fibers. It was 0.5 msec in duration and about 150 mv in amplitude. This stimulus resulted in activation of the myelinated B fibers as well as the nonmyelinated C fibers. Frequently, the initial small elevation, the B fiber activity, merged with the stimulus artifact. However, the main elevation represents the C fiber activity.²¹

After a nerve was found to have an action potential, it was irradiated and superfused simultaneously. It was vertically suspended by an attached thread some 3 cm from above into the center of a cylindrical UV lamp with 3 coils (PCQ-Xl-photochemical lamp, Ultra-violet Products, San Gabriel, Calif.). The thread suspending the nerve was secured through a rubber dropper fastened around the tip of the burette. The nerve was superfused dropwise at a constant rate with cold oxygenated Locke solution dripping from the burette onto the thread. Thereby, the nerve received steady and adequate perfusion dropwise along its entire length. With a second, lower thread tied to the bottom of the nerve, the perfusate continued vertically along its course which led into a beaker positioned beneath the cylindrical lamp.

The UV lamp which has 3 circular coils arranged vertically had an irradiation area of 3 inches in diameter and 5 inches in height. In the axis of the cylindrical cavity, according to the manufacturer's specifications, the intensity of the lamp is 30,000 $\mu\text{W}/\text{cm}^2$ of 254 m μ wavelength.

It was ascertained that about 2 hours of radiation were required to completely destroy the action potential in most instances.



Sometimes, however, with shorter periods of irradiation, e.g., 20 min, even in freshly removed nerves, the action potential seemed to disappear. But it was always restored within the hour just by allowing the nerve to sit in Locke solution (viz. historical background: findings of Audiat and Hutton-Rudolph).

Compounds used were Locke solution (as described above) and thiamine hydrochloride (Sigma Chemical Company).

Thiamine was assayed in nerves by the fluorometric method of Fujiwara and Matsui,⁴⁴ modified for microdetermination in the Turner fluorometer and also in the Aminco-Bowman spectrophotometer. Thiamine was extracted by homogenization of the nerves with 0.5 ml 5% TCA followed by centrifugation.

ATP levels were assayed by an enzymatic fluorometric technique developed by Lowry et al.²⁷



RESULTS:

(A) The destruction of the action potential:

Desheathed rabbit vagus nerves were irradiated for a period of about two hours under UV light (i.e., until the action potential was abolished), while being simultaneously superfused with oxygenated Locke solution. It should be noted, however, that with irradiation periods of 0.5 hour, the action potential was observed to disappear sometimes. The nerve was then removed from under the UV lamp. Spontaneously, the perfused nerve regained its action potential within an hour. Also, during these brief irradiation periods, with the nerves in a chamber isolated from further UV exposure, the minimum excitability voltage initially decreased and then increased after about twenty minutes (viz. discussion on Hutton-Rudolph, p. 3). At the beginning of these irradiation periods, the nerve's spike amplitude was also increased with irradiation. Similarly, Bachoffer²⁵ reported this phenomenon in single nerve fibers in earthworms. By discontinuing UV irradiation shortly after the nerve was responding in an enhanced manner, it was observed that the enhanced activity (increased spike amplitude, rate of rise of spike, and conduction velocity) was retained. However, after about twenty minutes the above increases were markedly reversed.²⁵

Successive trials in attempting to monitor restored conduction in these nerves after a 2-hour irradiation by returning them to Locke solutions were unsuccessful. At points in time ranging from immediately after the irradiation period up to twenty-four hours after, the B as well as the C fiber potential had been completely abolished (Fig. I, nerve #2). Perfused control (unirradiated) nerves maintained their



action potential some six to seven hours after being desheathed.

(B) The return of the action potential.

Employing the same procedure in the rabbit's second vagus, after the irradiation period we suspended action potential-deficient nerves in Locke solution containing 1 mM thiamine (Fig. I, nerve #1). In these thiamine-treated nerves we were able to restore both the B and C fiber activity in about one and one half hours to approximately 30-70% of its spike amplitude in 8 out of 9 experiments. Periodic monitorings at various intervals during the course of the experiment are illustrated in the figure below. The increasing amplitude with time in the B and C fiber potentials after irradiation in thiamine-treated samples may be noted.

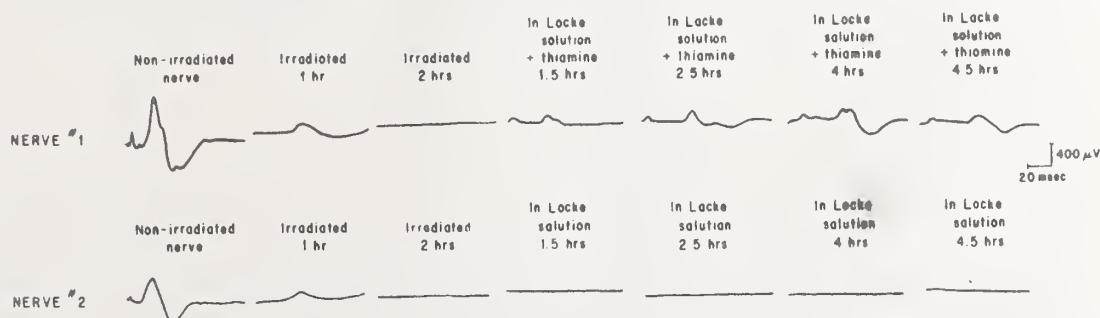


Fig. I. Effect of thiamine in restoring the action potential in UV-irradiated rabbit vagus nerves. Details in text.



Control nerve bundles of unirradiated nerves, when stimulated, discharged without decline for about six to seven hours after being desheathed. After this period there was a gradual decline in spike amplitude with cessation of depolarization after approximately twenty-four hours. (In one instance, a nerve restored in thiamine (after its action potential had been abolished by UV irradiation) retained its ability to discharge when stimulated some thirty-six hours later).

(C) Thiamine assays.

Using a filter fluorometer to assay for thiamine concentrations in a series of unirradiated desheathed rabbit vagi, we obtained an average thiamine content of 3.8 ng thiamine/mg of nerve sample. This value was in accord with previous determinations in this laboratory.

Table 1: Concentration of thiamine in ng/mg nerve

A. Unirradiated Nerves	B. Irradiated Nerves
2.8	0
4.1	0
3.5	0.38
<u>4.2</u>	<u>0.19</u>
Average:	3.6 ng Thiamine/mg nerve
	0.11 ng Thiamine/mg nerve

The presence of a fluorescent irradiation product was revealed by scanning the extract of irradiated nerves on an Aminco-Bowman spectrophotometer. Samples from action potential deficient nerves, treated only with NaOH and not CNBr, consistently had high fluorescent levels. These nerve samples, scanned on the Aminco-Bowman



spectrophotofluorometer, showed an emission spectrum with a maximum at about $465 \text{ m}\mu$ which is well within the thiamine range. Thiamine fluoresces at about $435 \text{ m}\mu$. Thus, we had evidence of a contaminant derived from radiation. This was corrected for in the assay as follows: one aliquot of the extract was assayed in the usual manner and this figure represented the fluorescence of both thiamine and the unknown material. In a second aliquot the addition of CNBr and NaOH was reversed and this figure reflected only the unknown fluorescent material (thiamine is destroyed by NaOH). By subtraction, then, one could determine fluorescence due to thiamine alone.

(D) ATP assays.

ATP levels were assayed in (i) control desheathed nerves, (ii) irradiated nerves, and (iii) irradiated nerves followed by thiamine treatment to restore the action potential. In the normal nerves, the ATP concentration was 2.48 mM but wide variations were found in irradiated nerves, both untreated and treated. Irradiated nerves, not in thiamine, had ATP concentrations ranging from 0.61 to 2.74 mM with a mean of 1.67 mM; irradiated, thiamine-treated nerves had values ranging from 0.05 to 5.45 mM with a mean of 1.98 mM. With these extreme variations, mean values are of little value. However, within each experiment using 2 vagi from the same rabbit, no significant difference in ATP content was observed regardless of whether the irradiated nerve was treated with the vitamin. This finding is compatible with the findings of unaltered thiamine dependent enzyme levels despite drastic changes in conductive ability.^{9,10}



(E) Ancillary finding.

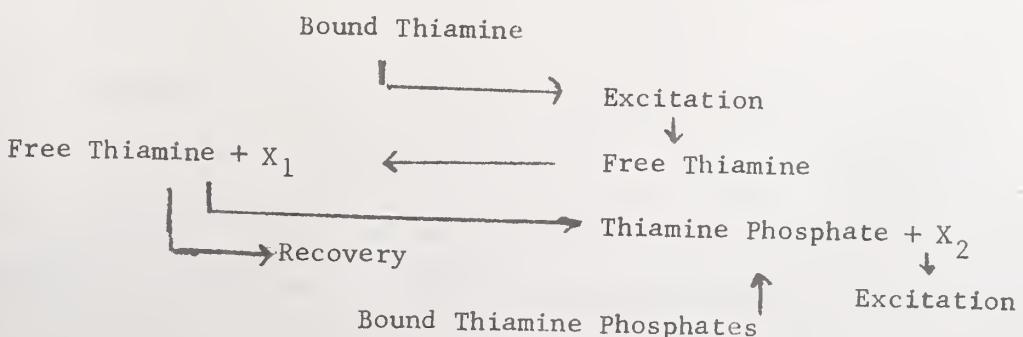
In one eight-hour-old desheathed nerve which had lost its action potential while in Locke solution all day, the full B and C fiber potentials were restored within about ten minutes after the nerve was treated with 1 mM thiamine in Locke solution.

(

DISCUSSION:

Our findings demonstrate that thiamine is essential to nerve conduction. UV irradiation during the course of about two hours resulted in the destruction of thiamine in the nerve membrane. Simultaneously, by fluorometric assay, thiamine concentrations were negligible as compared to those of control, unirradiated nerves. Only subsequent thiamine-treated nerves went on to conduct approximately 1.5 hours after irradiation. Control preparations in Locke solution only, failed to manifest any restoration of activity. Although the action potential of the thiamine-treated preparation rarely returned to its pre-irradiation level, even a partial return is significant as compared to the control nerve and in view of the manipulations that are involved in this procedure. Since no significant difference was observed in ATP levels between irradiated nerves in the presence and absence of thiamine this would imply that the thiamine effect had nothing to do with metabolism of the nerve but was strictly involved in the conduction process.

Various mechanisms have been suggested to explain the chemical basis of permeability changes which would implicate thiamine. Based upon his extensive work along these lines, von Muralt,³⁷ the originator of the idea that thiamine also acts neurophysiologically in nerves, presented the following scheme in 1958:





Thiamine is pictured, somehow, to commute between a "free" phase and a "bound" phase. The free thiamine and some unknown entity, X_2 , are a consequence of excitation. They ultimately result in additional "bound" thiamine phosphates with recovery, and in released thiamine phosphates with excitation. In essence, the mechanism was pointing to a phosphorylation and dephosphorylation process involving thiamine during the course of membrane depolarization and repolarization.

Along these latter lines in 1960 Petropoulos,²⁴ based upon the ionic hypothesis of electrical activity,⁴⁰⁻⁴¹ reasoned as follows: The rate of rise of the action potential, dx/dt , is a theoretical measure of the influx of Na^+ ions into the membrane. The S curve, (i.e., dx/dt plotted against membrane potential), obtained for a single nerve fiber treated with a thiamine antimetabolite, shows a decrease in the height in the upper plateau. This suggested to the author a decrease in the "number of active Na carriers." Since this reduction in the height of the action potential is abolished by addition of excess thiamine, "a loose carrier mechanism may be postulated for thiamine."²⁴

Itokawa and Cooper,²⁰ in view of the evidence summarized in the introduction and in their correlation of the effect of neuroactive drugs on the release of thiamine from nervous tissue, postulated "a carrier role for TPP or TTP involving a successive dephosphorylation and rephosphorylation of the vitamin as ion exchange takes place across the membrane." Complexes binding TPP and TTP with Na^+ and Ca^{2+} have been described by Hoffman et al.⁴² A second possibility which they offered, links thiamine with conformational changes in the membrane. In this case a shift of charged particles, similar to



the hypothesis of Baker,⁴³ "would induce a conformational change in the protein-lipid-thiamine phosphate mosaic of the membrane to displace the thiamine phosphate and permit a rapid influx of Na^+ and Ca^{2+} ."²⁰

All of the above mechanisms implicate thiamine in nervous tissue conduction but further work is necessary to dissect the events in conduction at a molecular level.



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